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BIOPHYSICS

Primary cilia as the nexus of biophysical and hedgehog signaling at the tendon enthesis

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The tendon enthesis is a fibrocartilaginous tissue critical for transfer of muscle forces to bone. Enthesis pathologies are common, and surgical repair of tendon to bone is plagued by high failure rates. At the root of these failures is a gap in knowledge of how the tendon enthesis is formed and maintained. We tested the hypothesis that the primary cilium is a hub for transducing biophysical and hedgehog (Hh) signals to regulate tendon enthesis formation and adaptation to loading. Primary cilia were necessary for enthesis development, and cilia assembly was coincident with Hh signaling and enthesis mineralization. Cilia responded inversely to loading; increased loading led to decreased cilia and decreased loading led to increased cilia. Enthesis responses to loading were dependent on Hh signaling through cilia. Results imply a role for tendon enthesis primary cilia as mechanical responders and Hh signal transducers, providing a therapeutic target for tendon enthesis pathologies.

INTRODUCTION

Tendon and its associated enthesis are essential components of the musculoskeletal system, linking muscle to bone to allow for coordinated muscle contraction, skeletal movement, and stability (1). This critical connection between muscle and bone, however, is prone to injury, accounting for a large number of musculoskeletal injuries in the United States every year (1–3). These injuries are often the result of overuse damage or underuse degeneration, leading to tendinopathy, enthesopathy, and eventually tissue rupture. Despite the prevalence of these injuries, no consensus treatments are available to prevent tendinopathy or enthesopathy or to recover a torn tissue to its native structure and full biomechanical functionality. There remains a critical gap in knowledge in our understanding of tendon enthesis responses to loading and the biological mechanisms that control these responses. The development of effective therapeutics is therefore hampered by our limited knowledge of tendon enthesis development biology, mechanobiology, and the endogenous mechanisms governing tendon pathogenesis and healing (3).

Both mechanical force and hedgehog (Hh) signaling are necessary for the development and maintenance of the tendon enthesis (4, 5). Loading deprivation of mouse shoulders during postnatal development causes structural, compositional, and functional defects in the tendon enthesis, including reduced collagen fiber alignment, decreased mineral content, and impaired mechanical function (6). Conversely, application of moderate in vivo loading by treadmill running in both adult and aging mice shows beneficial effects, including increased gene expression related to tenogenesis, enhanced mechanical properties, and altered tendon composition (7). Hh signaling also drives enthesis fibrocartilage formation, mineralization, and healing. A unique population of Hh-responsive cells build the tendon enthesis at the postnatal stage (5, 8, 9). Ablation of these cells resulted in decreased fibrocartilage formation and collagen disorganization. Conditional deletion of Smoothened (Smo), a transmembrane protein necessary for Hh responsiveness, resulted in thinner fibrocartilage and decreased biomechanical function of the tendon enthesis (5, 8, 9). Enthesis cells with high Hh activation are also involved in enthesis healing, suggesting their importance for enhancing healing outcomes (5, 10). Nonetheless, it has been a challenge to identify the cellular mechanism(s) controlling downstream cascades of mechanotransduction and the specific regulation of Hh signaling on tendon enthesis development and adaptation.

The primary cilium, an antenna-like nonmotile organelle that projects from the apical surface, is a critical component of intracellular biophysical and Hh signaling for many cells (11). Articular cartilage with disrupted primary cilia had increased thickness and reduced mechanical properties (12). Removal of primary cilia also markedly reduced loading-induced matrix deposition in chondrocytes (13). In bone, disruption of cilia formation in osteocytes resulted in a failure to trigger intracellular signaling cascades upon mechanical stimulation and a decrease in vivo bone formation (14). Furthermore, several components of the Hh signaling pathway, including Smo, are localized on or transduced inside the primary cilium itself, and Hh signaling activation and suppression are, in part, dependent on cilia (11). Mutation of ciliary proteins impairs Hh signaling, leading to ciliopathies such as cleft palate and polydactyly (15). These prior findings motivate our hypothesis that tendon enthesis formation and function are driven by cilia-dependent biophysical and Hh signaling. To test this hypothesis, we used tendon enthesis–specific mouse models and manipulated their physiological loading environments. We found that primary cilium incidence was temporally synchronized with Hh signaling during enthesis development. Enthesis cells responded to mechanical loading by inversely adjusting cilium incidence. Genetic disruption of cilia components led to defects in enthesis structure and mechanical properties. Furthermore, enthesis cell incidence was induced by the removal of Hh signaling and was coupled with diminished adaption to loading. Thus, our work revealed that primary cilia were at the nexus of mechanoresponsiveness and Hh signaling, directing tendon enthesis development and mediating its acclimatization to physiological loading.

RESULTS

Primary cilia are prevalent at the postnatal tendon enthesis

We previously demonstrated that Hh signaling is essential for tendon enthesis formation; furthermore, others have shown that primary cilia are critical for transduction of Hh signaling to musculoskeletal

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cell nuclei (5, 15). Therefore, we investigated tendon enthesis cell ciliogenesis during tendon enthesis development into maturity in a murine model. Antibodies against ciliary axoneme markers, including acetylated α-tubulin, IFT88 (Intraflagellar transport protein 88) (ciliary axoneme markers), and/or the centrosome marker pericentrin, were used for immunostaining to detect primary cilia of supraspinatus tendon enthesis cells from different developmental stages (Fig. 1 and fig. S1). Cilium incidence (the number of ciliated cells normalized by the total cell number) of tendon enthesis cells increased markedly in the first 2 weeks postnatally, coincident with the pattern of Hh activation

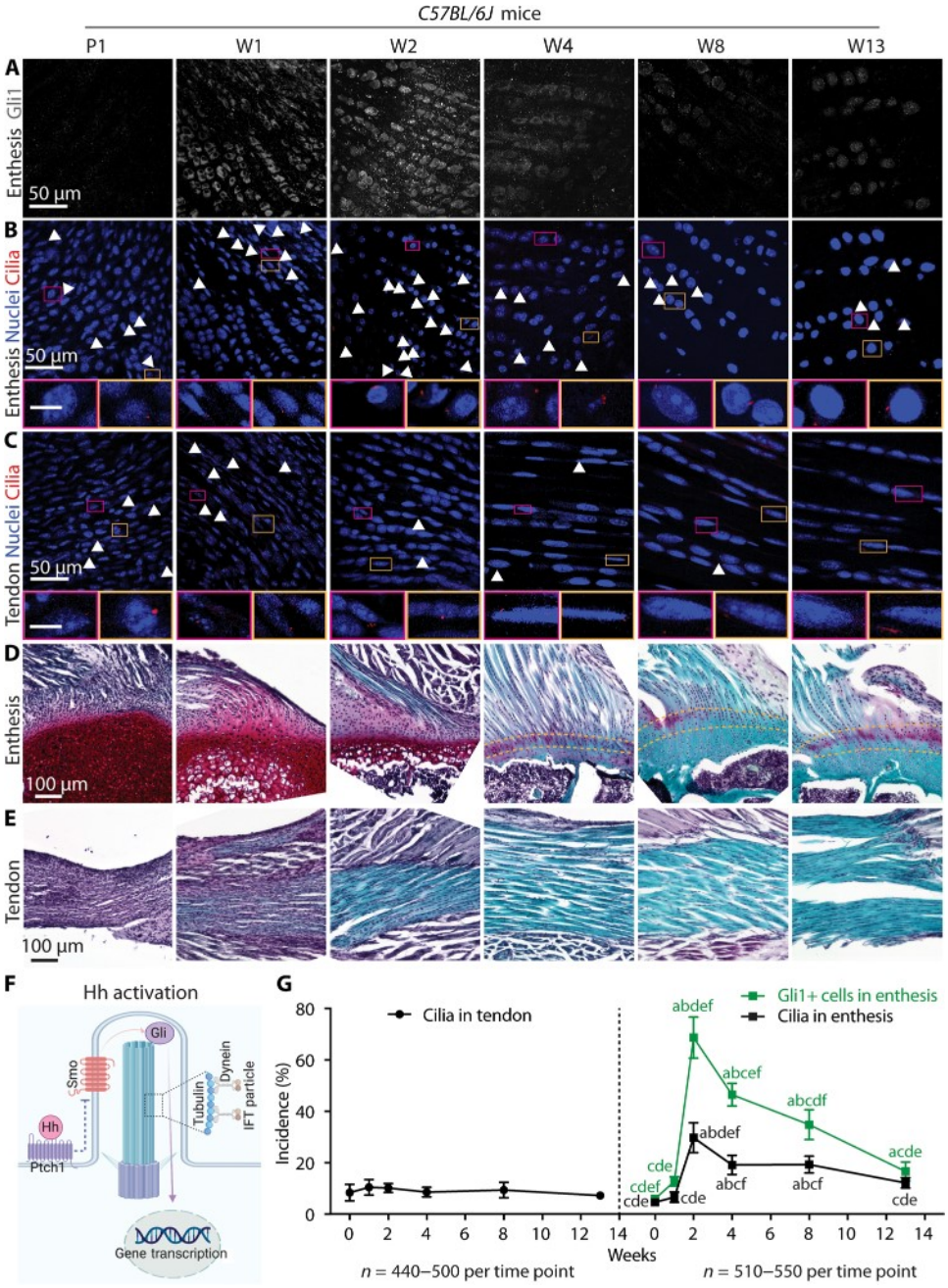

Fig. 1. Ciliogenesis of tendon enthesis cells during postnatal development coincides with Hh signaling and enthesis mineralization. (A to C) Immunofluorescence staining of Hh component Gli1 (gray) at the supraspinatus tendon enthesis (A) and primary cilia via acetylated tubulin (red, white arrowheads) at the enthesis (B) and midsubstance (C). Panels below (B) and (C) are magnified images corresponding to the colored rectangles. Arrowheads mark primary cilia; 4′,6-diamidino-2-phenylindole (DAPI) stains nuclei; P1, postnatal day 1; W1, postnatal week 1. Scale bars, 10 μm (panel insets). (D and E) Safranin O staining of the tendon enthesis and midsubstance at different developmental stages. Dashed lines mark the tendon enthesis. (F) Illustration of the transduction of Hh signaling inside the primary cilium. IFT, intraflagellar transport protein. (G) Quantification of the incidence of ciliated cells at the tendon and tendon enthesis as well as Gli1-positive (Gli1+) cells, normalized by the total cell number. Four to five mice were analyzed per time point; all data are represented as means ± SD; n represents the number of cells counted. a, $P < 0.05$ compared to W0; b, $P < 0.05$ compared to W1; c, $P < 0.05$ compared to W2; d, $P < 0.05$ compared to W4; e, $P < 0.05$ compared to W8; f, $P < 0.05$ compared to W13.

[i.e., glioma-associated oncogene (Gli1) expression] (Fig. 1, A, B, D, and G). In contrast, tendon midsubstance cells maintained a low level of ciliogenesis throughout postnatal development (Fig. 1, C, E, and G). The proportion of tendon entheses cells with cilia increased significantly between postnatal weeks 1 and 2 (W1 and W2), from 4.6 to 29.7%, and then decreased gradually to 12.1% by W13. Correspondingly, expression of Gli1 protein, the downstream effector of Hh signaling, changed in parallel with cell ciliogenesis during enthesis development (Fig. 1, A and G). Entheses cells had the highest activation of Gli1 and concurrent cilium incidence at W2 (Fig. 1G), coinciding with the onset of mineralization of the supraspinatus tendon enthesis (Fig. 1D) (16). These results imply that primary cilia contribute to Hh-driven and loading-regulated enthesis mineralization.

There is an inverse relationship between in vivo loading and primary cilium incidence

Motivated by the mechanosensory role of cilia in chondrocytes and osteocytes (17–19), we evaluated the connection between primary cilia incidence and tendon enthesis mechanoresponsiveness during postnatal development. Tamoxifen (TA)–inducible GliCreER^{T2};Rosa26^{mTmG} mice were used to track Gli1-lineage (Gli1-lin) cells. Mice were injected with TA at P14 (TA14) or P28 (TA28) and sacrificed at P56. Shoulders were paralyzed starting at birth using botulinum toxin A (BtxA) injection, a model that we previously established to unload the supraspinatus tendon enthesis and leads to defects in tendon enthesis formation (6). Compared to the entheses from contralateral control shoulders, unloading increased cilium incidence by 33% in TA14 mice and 66% in TA28 mice, respectively (Fig. 2, A to C). Similarly, unloading increased the Gli1-lin cell population by 48% in TA14 mice and 59% in TA28 mice (Fig. 2D). Unloading did not significantly change cilium incidence in the Gli1-lin cells in TA14 mice but resulted in a 31% decrease of cilium incidence in TA28 mice (Fig. 2E). However, cilium incidence of non–Gli1-lin cells was increased by 109 and 124% in TA14 and TA28 mice, respectively, after unloading (fig. S2). Unloading from birth through P56 led to significant up-regulation of genes related to ciliogenesis (e.g., IFT88, IFT80, and Dync2li1) and Hh signaling (e.g., Ptch1, Gli1, Gli2, and Gli3; Fig. 2F). These results demonstrate that biophysical forces drive ciliogenesis and activation of Hh signaling during enthesis development.

To determine mechanoresponsiveness of cilia in the adult enthesis, BtxA was used to unload tendon entheses, and treadmill running was used to overload tendon entheses (7). Consistent with the effects of unloading during development, 2 weeks of unloading led to increases of 166% in cilium incidence and 175% in Gli1-positive (Gli1+) cells (Fig. 3, A and C). Similarly, 4 weeks of unloading led to increases of 142% in cilium incidence and 128% in Gli1+ cells. In contrast to results during postnatal development, unloading of skeletally mature tendon entheses did not significantly change cilium incidence of Gli1+ cells, demonstrating that cilium assembly was not fully synchronized with Hh signaling in the adult tendon enthesis. In contrast to the effects of unloading, overloading led to a 48.7 and 56.8% decreases in cilium incidence after 2 and 4 weeks of treadmill running, respectively (Fig. 3, B and D). Running-induced overloading did not modify the population of Gli1+ cells nor did it change cilium incidence of Gli1+ cells. Overloading through 4 weeks of treadmill running led to down-regulation of genes related to ciliogenesis (i.e., IFT88, Kif3A, and Dync2li1) and Hh signaling (i.e., Gli2; Fig. 3E). In summary, unloading and overloading experiments in postnatal and adult animals demonstrate an inverse relationship between loading and cilium incidence/Hh activation.

Primary cilia are necessary for enthesis formation

The loading and unloading data demonstrate modulation of ciliogenesis at the tendon enthesis in response to in vivo loading. To extend our understanding of the role of primary cilia at the enthesis, we examined their necessity for enthesis formation. Tendon-specific (Scx-Cre) conditional deletion of IFT88, a ciliary gene, was achieved using ScxCre;IFT88$^{fl/fl}$ (cKO) mice (20). cKO mice had a 64% decrease in cilium incidence compared to their littermate controls (Fig. 4, A and E). Phenotype analysis from P10 to W13 demonstrated clear effects of cilia disruption on enthesis formation. The wild-type (WT) entheses had rounder and apparently larger cells compared to cKO, consistently from P10 to W13 (Fig. 4, B and C). This alteration in fibrocartilage cell phenotype was confirmed by changes in the expression of collagen X, a marker for hypertrophic chondrocytes; collagen X expression was significantly decreased by 67% in entheses from cKO mice compared to entheses from WT mice at 8 weeks (Fig. 4, C and F, and fig. S3D). Cortical and trabecular bone were not affected by IFT88 knockout until W13 (Fig. 4G). At this time point, cKO mice had significantly deteriorated bone morphometry of the humeral head, with thinner cortical bone and less mineralized enthesis fibrocartilage (Fig. 4, C and G, and fig. S2, C and F). Furthermore, the tendon entheses from 13-week-old cKO mice had decreased structural properties (i.e., maximum force and stiffness) and increased material properties (i.e., stress and modulus; Fig. 4H), with drastically smaller cross-sectional areas in cKO tendon entheses (fig. S3A). IFT88 knockout in Scx-expressing cells led to overall changes in mouse physiology. The growth of cKO mice was slower, with significantly lower body weights compared to WT controls (fig. S3A and B). Thirty percent of cKO mice died between W6 and W13, possibly due to polycystic kidney disease directly caused by deletion of IFT88. Loss of IFT88 in Scx-expressing cells resulted in altered mouse locomotion, e.g., swing speed and cadence (fig. S3A).

Ciliary Hh signaling mediates mature enthesis adaptation to in vivo loading

To examine the interaction between primary cilia, Hh signaling, and in vivo mechanical loading, we generated mice that harbored a tendon-specific loss of the Hh receptor Smo (Smoothened) and subjected them to unloading and overloading protocols (Fig. 5A). Deletion of Smo caused a 55.0% increase in tendon enthesis cells with primary cilia, indicating that cilia assembly was maintained or induced by Hh disruption (Fig. 5, B and D). Overloading and unloading also induced cilia disassembly and assembly, respectively, in the entheses from ScxCre;Smo $^{fl/fl}$ cKO and WT mice, further demonstrating a mechanosensory role of cilia at the enthesis (Fig. 5D). Consistent with our previous study, loss of Smo in Scx-expressing cells led to a loss of enthesis fibrocartilage and decreased structural and material properties (Fig. 5, C and E to G, and fig. S4A). When considering enthesis mechanoresponsiveness, both overloading and unloading led to changes in enthesis mineralization (i.e., increased and decreased densities of cortical bone after overloading and unloading), bone morphometry, and mouse gait in WT mice (Fig. 5 and fig. S4). In contrast, Hh deletion mitigated many of these loading-induced changes to the tendon enthesis of WT mice: cross-sectional area in WT mice decreased after both overloading and unloading (Fig. 5E), stiffness and ultimate stress decreased after unloading, and ultimate

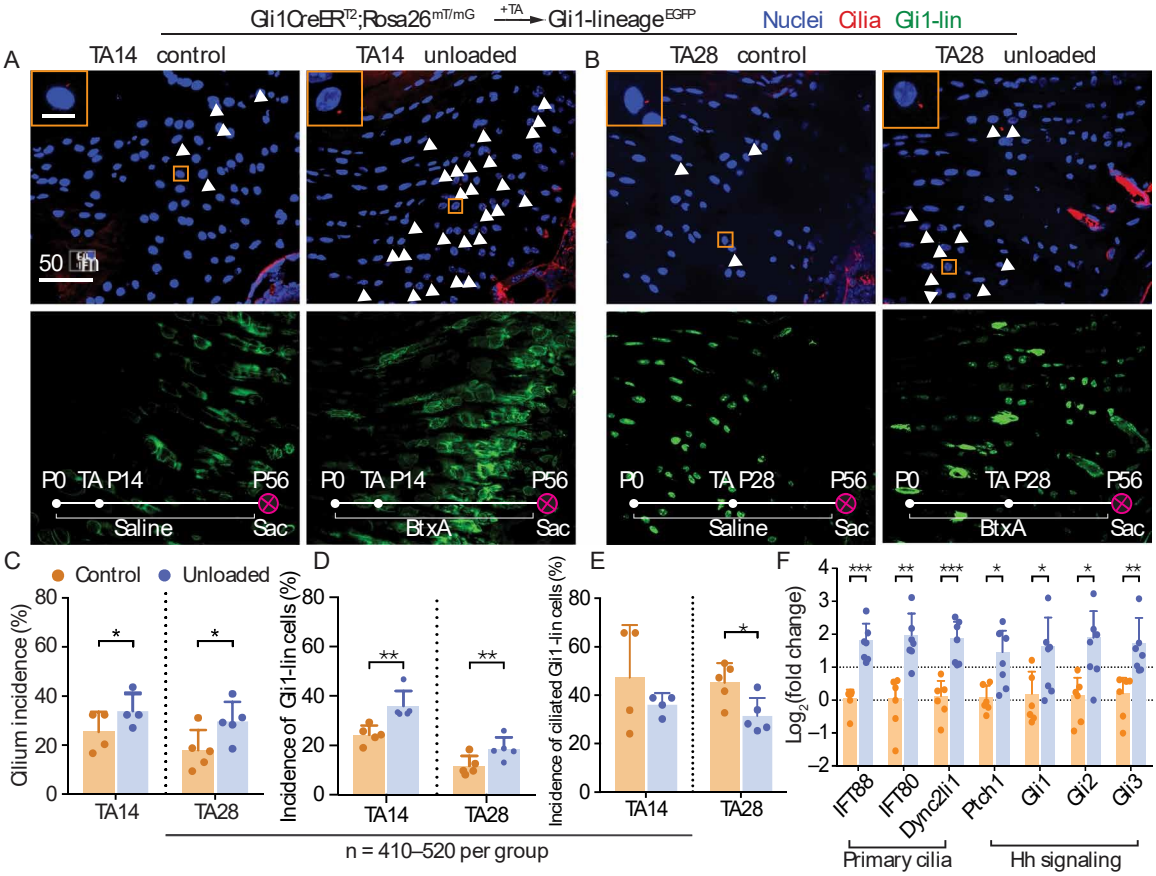

Fig. 2. Unloading during tendon enthesis development stimulates primary cilia assembly. (A and B) Immunofluorescence for primary cilia (red, white arrowheads) of the tendon enthesis from Gli1CreER^{T2};Rosa26$^{mT/mG}$ mice with TA injection and BtxA-induced paralysis (unloaded, right shoulder) or saline injection (control, left shoulder) at the time points indicated. Panels on the top left show magnified views of the orange rectangles. DAPI stains nuclei; P0, postnatal day 1; TA, tamoxifen injection; Sac, sacrifice; scale bar, 10 μm (inset). (C) Incidences of ciliated cells per all the cells at the tendon enthesis of (A) and (B) mice with TA injection at P14 and P28. n represents the number of cells counted. The error bars show SD of tissue samples from different animals. *P< 0.05, **P< 0.01, ***P< 0.001. (D) Incidences of Gli1-lin cells per all the cells at the tendon enthesis of (A) and (B) mice. (E) Incidences of ciliated Gli1-lin cells normalized by Gli1-lin cells at the tendon enthesis, showing the incidence of Gli1-lin cells that were ciliated. (F) Expression of genes related to primary cilia and Hh signaling from tendon entheses of 8-week-old C57BL/6J mice paralyzed since birth.

stress increased after overloading; none of these outcomes were affected by loading in ScxCre;Smo$^{fl/fl}$ cKO mice (Fig. 5F). When examining the morphometry of bone adjacent to the tendon enthesis, overloading increased and unloading decreased cortical bone densities (tissue mineral density) and trabecular bone densities (bone mineral density) in both genotypes (Fig. 5G). Similar results were seen in other bone morphometry outcomes (fig. S4B). At the whole animal level, both swing speed and cadence were increased by unloading for both genotypes (Fig. 5H and fig. S4C).

DISCUSSION

The current study demonstrated that tendon enthesis formation and function are driven by primary cilium-dependent biophysical and Hh signaling. We first identified that the primary cilium is critical for tendon enthesis mechanoresponsiveness. Ciliogenesis during enthesis development and maturity was inversely driven by physiological loading. In addition, cilium incidence at the tendon enthesis increased during enthesis mineralization and was synchronized with Hh signaling, supporting the concept of interplay between Hh signaling and cilia (21). This is consistent with previous reports that both primary cilia and Hh components change dynamically during muscle differentiation (22). Further evidence of this interplay was shown through deletion of Hh signaling, which caused abnormal ciliogenesis and diminished mechanoresponsiveness at the tendon enthesis. Most markedly, the deletion of primary cilia from the tendon enthesis caused significant structural and compositional defects to the enthesis. Therefore, primary cilia are key regulators of enthesis formation and maintenance, modulating their assembly in response to biophysical forces and their interaction with Hh signaling.

Consistent with the current in vivo results, an inverse relationship has been demonstrated previously between ciliogenesis and mechanical loading in vitro (17, 23, 24). Mechanical stimulation above a threshold leads to cilia shortening and disassembly, and removal of mechanical loading leads to cilia elongation and assembly (24–26). This response may be an effort of cilia to scale their sensitivity according to the loading signal. Cilium elongation or assembly increases the mechanosensitivity of the cilium or increases the number of mechanosensors on the ciliary membrane, respectively, which improves the cell's ability to sense mechanical cues from its environment